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TITLE: Neurocognitive and Biomarker Evaluation of Combination mTBI from Blast Overpressure and Traumatic Stress

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14. ABSTRACT

Mild traumatic brain injury (mTBI) and post-traumatic stress disorder (PTSD) are major medical issues for the Warfighter. The current project is designed to evaluate the impact of mild traumatic brain injury (using blast overpressure) and the processes involved in traumatic stress (using a predator exposure procedure and a conditioned fear procedure) in a rodent model. The studies evaluate these insults alone and in combination to specifically address the question of whether mTBI can exacerbate the effects of psychological stress. Additionally, following the insults, a molecular biological evaluation is performed based upon the discovery of biomarkers that have been shown to be correlated with other forms of TBI. Thus, the project aims to systematically assess the combined effects of blast overpressure, traumatic stress and learned stress responses in rodents with the aim of understanding how these forces may interact to impact behavior as well as evaluating their outcome on known biomarkers involved in TBI and stress response system activation.

15. SUBJECT TERMS
None provided.

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INTRODUCTION:

Mild traumatic brain injury (mTBI) and post-traumatic stress disorder (PTSD) are medical issues for the war fighter. Sometimes, mTBI and PTSD present a convergence of symptoms, making it difficult to distinguish between the behavioral manifestations of the two conditions and to determine the extent to which the processes of traumatic stress and mild brain injury might be related. The current project is designed to evaluate the impact of these two insults in rodent models. To model the effects of mTBI, we are using a blast overpressure (BOP) procedure. Two different procedures are used to model traumatic stress / PTSD. First, a predator exposure procedure is used to present a traumatic stress event to the rat; second, a conditioned fear procedure is used to model a process known to be disrupted in PTSD. Notably, the studies evaluate these insults alone and in combination to specifically address the question of whether mTBI can exacerbate the effects of psychological stress. The studies are focused on evaluating the short- and long-term behavioral impacts from the insults, and use dependent measures from procedures including operant performance, conditioned suppression (conditioned fear), Morris water-maze and elevated plus maze. Following the insults and the behavioral testing, a molecular biological evaluation is performed based upon the discovery of biomarkers that have been shown to correlate with other forms of TBI. Thus, the project aims to systematically assess the combined effects of blast overpressure, traumatic stress and conditioning responses in rodents. The overall aim of the project is to increase our understanding of how these challenges interact to impact behavior and how they are reflected in known biomarkers involved in TBI and stress response system activation.

BODY:

The project award date was 01SEP10. The project includes a partnering PI (Dr. Stephan Ahlers, NMRC) under a separate award (W81XWH-10-2-0091). A three-way cooperative research and development agreement (CRADA) between the Army (WRAIR), the Navy (NMRC) and the Geneva Foundation was negotiated and approved on 01MAR11. The CRADA was a required step before work on the study could progress. We have applied for and received preliminary approval for a no-cost extension to complete all work.

The project is broken up into three tasks, with a subtask under Task 2 and Task 3. The status of each task is given below.

Task 1: Generation of approved IACUC protocols.

Two protocols have been written and approved by the WRAIR/NMRC IACUC and by the ACURO oversight body. Essentially, one of the approved protocol covers all of the work in Task 3 (PI: Genovese, approved during last reporting period) and the second protocol (PI: Ahlers, approved during this reporting period) covers all of the studies for Task 2. All work on this task has been completed.

Task 2: Evaluation of combination BOP and predator exposure on (a) Morris water maze (n=40) and (b) elevated plus maze (n=40) with subsequent biomarker assay.

All behavioral work for the combination BOP and predator exposure evaluated with the Morris water maze (n=40) (Task 2a) has been completed.

Biospecimen collection from animals in Task 2a (n=40) is complete and sample processing is underway.

The final study in Task 2 is the evaluation of combination BOP and predator exposure evaluated with the elevated plus maze (Task 2b). This work is scheduled, but has not yet started.

Task 3: Characterization of BOP on conditioned fear with subsequent biomarker evaluation (total number of rats=60).

To address the issue of whether mTBI can modify a conditioned fear, we designed and implemented a conditioned fear procedure that is embedded in an operant behavior task. That is, rats are first trained to lever-press for food reinforcement under a variable-interval 32 second schedule of food reinforcement (VI32). The schedule produces a relatively constant rate of responding (lever pressing) throughout the 30 min test sessions. Once VI32 performance is stable, inescapable electric shock (IES or Unconditioned Stimulus [US]) is paired with a conditioned stimulus (CS) consisting of flashing lights and a pulsing tone. The pairings take place in a different chamber as does the VI32 task and can reasonably be considered to constitute a different environmental context. Subsequently, the CS is presented during the VI32; one presentation every 7 days after initial CS+US pairing, for a 56-day period (i.e., 8 weekly presentations). Subsequently, subjects are sacrificed for tissue harvesting and biomarker evaluation. Four treatment groups are employed: IES (CS+US pairings) + BOP (I-B), sham IES (CS only) + BOP (S-B), IES (CS+US pairings) + sham BOP (I-S), and Sham IES (CS only) + sham BOP (S-S).

This task consists of two studies. In the first (3a) (n=40), BOP and conditioned fear treatments are examined for 8 weeks following initial treatments with subsequent biomarker evaluation. In a second study (3b) (n=20), the same combinations of treatments (BOP and conditioned fear) are examined, but rats are sacrificed within 24 hours after the initial treatments with subsequent biomarker evaluation. Thus, the latter study is similar to the former study, but was designed to maximize the detection of biomarkers which might only appear shortly after the treatments. Both studies under this task consist of a number of subtasks and our progress under each subtask is detailed below.

Subtask 1: Acclimation / food restriction. Completed during last reporting period (n-60).

Subtask 2: VI32 acquisition. Completed for all rats (n=60).

Subtask 3: BOP / IES treatments. Completed for all rats (n=60).

Subtask 4: CR (CER) evaluations. Completed for all rats (n=60).

Subtask 5: Tissue harvesting and proteomic analysis. We have completed tissue harvesting for this task. We have completed rigorous method development for targeted analysis of biomarkers including GFAP, UCH-L1, SBDPs, PSD-95, nNOS, MBP, Syntaxin-6, p43/EMAPII, and MAP2 by Western blotting or ELISA for rats from Task 3b (n=20). Targeted analysis of biomarkers in biosamples for rats from Task 3a has been initiated. We have completed method development for proteomics analysis for which sample LC-MS/MS has been initiated. Results for a number of rats from Task 3b are included below.

Results and Preliminary Results

Behavioral evaluations from Task 3 evaluating the effects of mTBI on conditioned fear have been completed. These results have been submitted and accepted for publication (in press) and that manuscript is attached in lieu of a lengthy description of the results and their interpretation in this section. Additionally, preliminary analyses appeared in last year's annual report. Conclusions from this study appear in the section below.

Behavioral results from Task 2 (a) are to appear in the Partnering PI (Dr. Stephan Ahlers) annual report and the reader is referred to that document.

Proteomic results:

In-progress results described below address key aims of the project but are based on a partial set of subjects (as indicated). Although statistical analyses have been employed, it is essential to understand that some of these results are preliminary and are subject to change as the studies are completed and further analyses are performed. Results for GFAP, UCH-L1, SBDP-145/150 and SBDP-120 in tissues as well as preliminary results for GFAP and UCH-L1 in serum were reported in the prior quarterly reports and are re-iterated below for reference. Preliminary data for PSD-95, nNOS, MBP and syntaxin-6 are introduced.

Figure 1 shows the average quantitative abundance of TBI markers of injury GFAP and UCH-L1 after IES with or without BOP exposure in tissue samples. Brain tissue clarified lysates were analyzed by ELISA. Analyses identified no significant changes for any group in GFAP (A) or in UCH-L1 abundance (B) in all the brain regions studied.

Figure 2 shows the semi-quantitative abundance of α-II spectrin break-down products (SBDP) in tissue samples. SBDP-145/150 and SBDP-120 are produced by necrotic calpain-2 cleavage or apoptotic caspase-3 of neuronal α-II spectrin, respectively. SBDP-145/150 (A) and SBDP-120 (B) are graphed individually. There

were no significant differences in SBDP-145/150 between treatment groups in all of the brain regions studied. In contrast SBDP-120 was significantly increased by nearly 60% in the PFC after I-B compared to S-S.

Figure 3 shows the semi-quantitative abundance of PSD-95, a major dendritic spine protein, as an indicator of neuronal dendritic spine injury after fear conditioning and mTBI from BOP. PSD-95 was decreased by ~40% in the hippocampus in I-B group as compared to sham controls (S-S). There were no significant differences in nNOS between groups in other brain regions studied.

Figure 4 shows the semi-quantitative abundance of neuronal nitric oxide synthase (nNOS), a key binding partner of PSD-95 which is also enriched in dendritic spines. NNOS was decreased by ~30% in the hippocampus and by ~ 40% in the cerebellum in I-B group as compared to sham controls (S-S). There were no significant differences in nNOS between groups in other brain regions studied.

Figure 5 indicates preliminary semi-quantitative abundance of myelin basic protein (a marker and main constituent of oligodendrocytes which myelinate axons). Changes in MBP are often associated with TBI. Analysis indicated an increase in MBP in I-B compared to S-S within the cerebellum. No other changes between groups or other regions were statistically significant.

Figure 6 shows preliminary data of syntaxin-6 abundance in brain tissues. Syntaxin-6 is involved in intracellular vesicular trafficking. The average abundance was relatively unchanged. Analyses identified no significant differences across groups or brain regions.

Figure 7 shows data of TBI serum biomarkers 24h after injury measured by quantitative ELISA. The average abundance of GFAP and UCHL-1 after IES with or without BOP exposure in serum samples from rats from Task 3b is indicated. Analyses identified no significant changes in GFAP (A) or UCH-L1 (B).

Biomarker quantitation in the remaining serum and CSF will continue to be conducted with customized ELISA assays, which are prepared upon request with quality control by our collaborators (Banyan Biomarkers, Inc.). Proteomics analysis of brain tissue will continue with the study of differential protein abundance in the CTX in rats from Task 3b conducted in triplicate. All bio-fluids and tissues have been collected to continue these studies for Task 3b. We are currently in the process of continuing testing brain and serum biomarkers with remaining biosamples. For proteomics analysis, brain tissue sample preparation is complete and the first step in analysis by LC-MS/MS is in progress.

KEY RESEARCH ACCOMPLISHMENTS:

- Gained WRAIR/NMRC and ACURO approval for animal use protocol.

- Completed the behavioral evaluation of the effects of mTBI on the maintenance and extinction of a conditioned fear in rats.

- Developed and implemented a model to evaluate the combined effects of mTBI from BOP and predator stress evaluated using a Morris Water Maze in rats.

- Completed the tissue biomarker profile for GFAP, UCH-L1, SBDP-145/150 and SBDP-120.

- Continued analysis of PSD-95 and nNOS in brain tissues and of GFAP and UCH-L1 in serum.

- Initiated and continued analysis of MBP and syntaxin-6 in brain tissues.

- Completed rigorous method development and initiated LC-MS/MS analysis for proteomics of brain tissues. Biological replicates (N = 3) were parsed into 10 fractions each. Triplicate LC-MS/MS analysis for each fraction and database searching is in progress.

REPORTABLE OUTCOMES:

R.F. Genovese, L.P. Simmons, S.T. Ahlers, E. Maudlin-Jeronimo, J.R. Dave, A.M. Boutte, Effects of Mild TBI from Repeated Blast Overpressure on the Expression and Extinction of Conditioned Fear in Rats, Neuroscience (2013), doi: http://dx.doi.org/10.1016/j.neuroscience.2013.09.021

CONCLUSION:

With regard to the behavioral studies in Task 3, the major question has been:

Can mTBI from BOP affect conditioned fear?

We have shown that, in rats, a behavioral deficit in the expression of conditioned fear can be caused by mTBI from BOP. While the BOP produced a deficit in the expression of a conditioned fear, the deficit was observed as a reduction in the impact of the conditioned fear as compared to IES+sham (I-S) controls. Extinction to the conditioned fear appeared to occur normally in both IES+BOP (I-B) and IES+Sham (I-S) groups. It is notable that these results, demonstrating that the mTBI produced a reduction in the expression of a conditioned fear while not increasing resistance to extinction, are not in the direction of an effect that is analogous to PTSD.

While affecting the conditioned fear, the BOP did not produce acute deficits on general performance as measured by the VI, even when evaluated just a few hours after BOP exposure. Furthermore, no delayed effects on the VI were caused by the mTBI as no systematic changes in long-term performance on the VI were observed over two months of post-mTBI evaluations. In this regard, the results suggest that the intensity of the mTBI was, indeed, mild and is likely at the lowest end of the mTBI continuum.

With regard to the proteomic aspects of the studies, the discussion below is based on a partial set of subjects. Although statistical analyses have been employed in most cases, it is essential to understand that these results and the conclusions are preliminary and are subject to change as the studies are completed and further analyses are performed.
One of the major specific aims of this project is to address the question:

Are there biomarkers or other proteomic changes due to mTBI from BOP, with or without fear conditioning?

Many studies have observed that the proteins included in this study are possible markers of TBIs in clinical studies and animal models (Lumpkins, Bochicchio et al. 2008; Metting, Wilczak et al. 2012; Mondello, Robicsek et al. 2010; Pike, Zhao et al. 1998; Pike, Flint et al. 2001; Williams, Wei et al. 2007). Our preliminary data suggest that some of these proteins may also be differentially abundant in the study from Task 3b.

Surprisingly, GFAP and UCH-L1 were not significantly altered in any of the brain regions studied. Serum UCH-L1 and GFAP also did not indicate a clear change as a consequence of IES or BOP based on statistical analysis of preliminary data. However, other proteins included showed distinctly different abundance profiles.

Cleavage of alpha-II spectrin by caspase-3 and calpain-II generate fragments SBDP-120 and SBDP-145/150, respectfully, during cell death SBDP-120 was increased in the PFC after BOP, but was only significant with the combination of IES + BOP. The SBDP-145/150, a fragment generated by calpain-2 cleavage, showed non-significant increases in the PFC. The trend suggests that BOP may be the leading cause of the observations, since BOP with or without IES produced higher values compared to IES alone. Furthermore, these results infer that caspase, not calpain, driven cell death is prominent in the PFC 24h after IES with BOP.

Alteration of the post-synaptic density is associated with TBI (REF). PSD-95 and nNOS are not only binding partners, but are major constituents of healthy synaptic density or dendritic spines. PSD-95 and nNOS were decreased after IES + BOP in the

HP and nNOS alone was decreased in the CB after IES + BOP. These results may indicate spine retraction and/or spine remodeling after both forms of injury.

The other two proteins described, MBP and syntaxin-6, are markers of white matter (axonal) damage and vesicular trafficking, respectively. Preliminary data indicated that MBP increased after IES + BOP, which may infer a very small, but significant damage to axons in the CB. Syntaxin-6 was not affected by any combination of IES and BOP.

In addition to completing analyses of tissues from Task 3b, we will soon begin to evaluate select biomarkers in tissue samples from rats from Task 3a, which were collected ~8 weeks after IES and BOP. For these studies, we will take a rigorous, targeted approach to determine the best targets to study. These samples will allow for a temporal comparison of specific protein changes observed in samples from Task 3b.

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Mondello, S., S. A. Robicsek, et al. (2010). "alphaII-spectrin breakdown products (SBDPs): diagnosis and outcome in severe traumatic brain injury patients." J Neurotrauma 27(7): 1203-1213.

Pike, B. R., J. Flint, et al. (2001). "Accumulation of non-erythroid alpha II-spectrin and calpain-cleaved alpha II-spectrin breakdown products in cerebrospinal fluid after traumatic brain injury in rats." J Neurochem 78(6): 1297-1306.

Pike, B. R., X. Zhao, et al. (1998). "Regional calpain and caspase-3 proteolysis of alpha-spectrin after traumatic brain injury." Neuroreport 9(11): 2437-2442.

Williams, A. J., H. H. Wei, et al. (2007). "Acute and delayed neuroinflammatory response following experimental penetrating ballistic brain injury in the rat." J Neuroinflammation 4: 17.

APPENDICES:

A1. Acronym and Abbreviation Definitions

BOP: Blast overpressure. In our procedure, we are using three exposures at 75 kPa (~10.8 psi).

CER: Conditioned emotional response. With regard to the conditioned fear procedure, it refers to the conditioned response (CR).

CR: Conditioned response. The response elicited by the CS alone following pairing with a US. In the conditioned fear procedure, the conditioned response is assumed to include "fear."

CS: Conditioned stimulus. With regard to the conditioned fear procedure, it refers to the flashing lights and pulsing tone stimuli that are paired with IES initially and subsequently presented alone in the VI32.

CSF: Cerebral spinal fluid.

CTX: Cerebral cortex.

GFAP: Glial fibrillary acidic protein.

HP: Hippocampus.

I-B: IES + BOP. The treatment condition where rats receive the CS paired with IES (the US) and BOP.

I-S: IES + sham BOP. The treatment condition where rats receive the CS paired with IES (the US) and sham BOP.

IES: Inescapable electric shock. In our procedure, the CS is paired with the IES, which constitutes the US, to produce the fear conditioning.

mTBI: Mild traumatic brain injury. In our project the mTBI is produced by the BOP.

PFC: Prefrontal cortex.

S-B: Sham IES + BOP. The treatment condition where rats receive the CS only (no IES / US) and BOP.

S-S: Sham IES + sham BOP. The treatment condition where rats receive the CS only (no IES / US) and sham BOP.

SBDP: Spectrin break-down product.

SI: Suppression index. A measure to evaluate the degree of response suppression on the conditioned fear procedure, i.e., a measure of the magnitude of the CER. Calculated by the formula: (response rate before-response rate after) / (response rate before + response rate after). A suppression index is usually calculated for 1- and 3-min intervals before and after presentation of the CS.

UCH-L1: Ubiquitin carboxy-terminal hydrolase-L1.

VI32: Variable-interval 32 second schedule of reinforcement. The operant conditioning schedule specifying that one lever press following an average interval of 32 sec produces reinforcement. Individual intervals are normally distributed around a mean of 32 seconds.

A2: Methods and Procedures

The project includes a partnering PI (Dr. Stephan Ahlers, NMRC) under a separate award (W81XWH-10-2-0091). See Ahlers annual report for behavioral methods for Task 2a.

Detailed behavioral methods for Task 3 are contained in the attached research report and also in last year's annual report.

Detailed methods for biomarker evaluation appear below.

1.0. Tissue Harvesting.

1.1. Euthanasia.
A single-dose mixture containing 70 mg/kg ketamine and 6 mg/kg xylazine was administered to the rat via intramuscular injection using a 24-26 gauge needle. The dose generally induces deep anesthesia within 5 min and lasts for about 60 min, making it the preferred anesthetic agent for trans-cardial blood and cerebral spinal fluid (CSF) collection. Before making any incisions, an adequate level of anesthesia was verified by checking for loss of consciousness and failure to react to a noxious stimulus, such as a pinch on the tail with a pair of forceps. In all rats, blood and CSF samples are taken for biomarker analysis. Additionally, after euthanasia, brain tissue from each rat is collected for potential proteomic analysis.

1.2. Biosample collection.
For CSF collection, a 4-cm midline incision is made from 0.5 cm anterior to the interauricular line. The atlanto-occipital dura mater is exposed by separating the nuchal muscles and CSF is collected through by a 30 G syringe needle through the skull. Blood is collected by cardiac puncture. Both CSF and blood samples are collected into heparin coated tubes in the presence of protease/phosphatase inhibitors and stored on ice. A separate cohort of blood is collected in serum clotting tubes for 30 minutes. Serum is transferred to clean tubes and supplemented with protease/phosphatase inhibitors. All biofluids are centrifuged at 1200 g for 10 min at 4°C. The resulting plasma, serum, or clarified-cell free CSF is transferred to Eppendorf tubes and stored at -80° C. Select brain regions (prefrontal cortex, cerebral cortex, midbrain hippocampus, and cerebellum) of both left and right hemispheres are dissected, flash frozen in N2 (l), and individually stored at -80 °C until processing.

1.3. Western Blotting of Brain Tissues.
Brain tissues from the left hemisphere are sonicated for 2 X 10 s in 1 X RIPA lysis buffer containing protease and phosphotase inhibitors (Sigma, St. Louis, MO) and centrifuged at 10 kg, 4º C, 10 minutes. Clarified supernatant is collected and protein concentrations are determined by using the BCA protein assay kit (Thermo/Pierce, Rockford, IL). Samples containing 10 µg of protein are denatured, reduced with dithiothreitol (DTT), loaded and separated by 4–15% gradient polyacrylamide gel electrophoresis (PAGE) with the NuPage

system (Invitrogen, Grand Island, NY). After transferring to PVDF membranes, blots are probed with primary antibodies to each protein biomarker. Densitometry of protein band intensity is measured using an ImageQuant LAS 4000 with automated background subtraction (GE Healthcare, Piscataway, NJ). To compare biomarker abundance across multiple blots, 2µg total protein from a positive control is loaded into each gel as an internal control for gel-gel variation.

1.4. Enzyme Linked Immunosorbent Assays.

Protein samples extracted from brain tissues are prepared as with Western blotting, but normalized to contain 10 µg of total protein and 0.25 X RIPA per well. Tissue GFAP or UCH-L1 is determined using commercially available kits containing internal standards as described by the manufacturer (USCNK/Life Science; Cedarlane Laboratories, Burlington, NC). All biological samples are measured in duplicate using a colorimetric plate reader (450nm).

1.5. Biofluid Preparation.

Equal volumes of CSF (60uL) or serum (35uL) are diluted 10-fold and albumin and immunoglobulins are depleted using ProteoExtract resins (Millipore). Eluted protein solutions are concentrated with 3 kDa MWCO spin filters (Millipore) and the final volumes normalized. Total protein content in serum is estimated with the BCA kit (Thermo/Pierce). Serum is further normalized by volume and protein concentration prior to analysis. All samples are stored at -80°C until testing. These samples are tested for biomarkers using custom ELISA designed in-house.

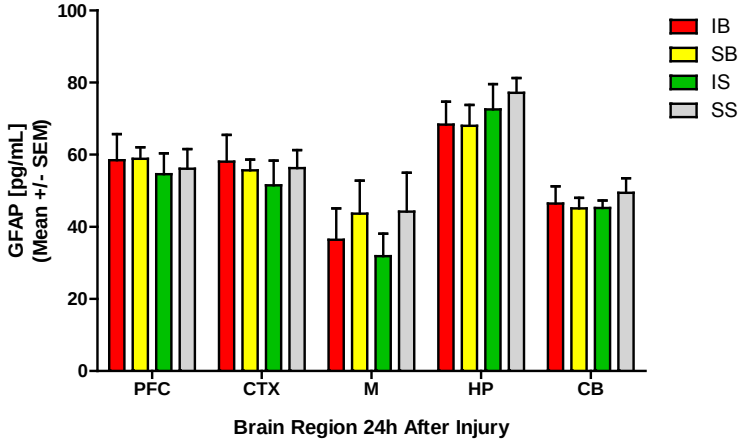
1.6. Proteomics Analysis of Brain Tissues.

To optimize protein detection and differential protein analysis, we have modified the proteomics protocol slightly from our original plan. Samples are prepared as stated for Western blotting and 20ug is loaded per sample per lane. After reducing and denaturing – PAGE, gels are stained with Colloidal Blue dye (Invitrogen, Carlsbad, CA). Protein bands are excised and pooled by molecular weight ranges. Each gel-piece containing protein is reduced, alkylated, and then digested with trypsin. The resulting peptides are extracted with acidified 50% acetonitrile, dried, and stored at -80 °C. Lyophilized peptides are re-constituted and then analyzed by shotgun proteomics. Differential protein abundance due to treatments is determined by spectral counting. Proteins that are significantly increased or decreased will be confirmed with mass spectrometry or immune-based methods such as Western blotting or ELISA.

SUPPORTING DATA:

Note: Treatment Groups are as follows: I-B, IES + BOP; S-B, Sham IES + BOP; I-S, IES+Sham BOP; S-S, Sham IES + Sham BOP.

Figures and Figure Legends



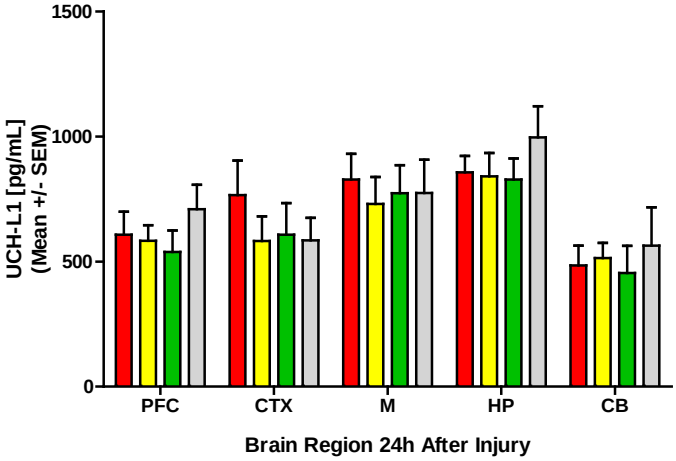


Figure 1. Quantitative ELISA of traumatic brain injury biomarkers (top, GFAP, bottom, UCH-L1) after fear conditioning and mTBI from BOP. Tissue samples are from rats in

Task 3b and were sacrificed 24 h following the last BOP exposure. Proteins were individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (PFC), cerebral cortex (CTX), midbrain (M), hippocampus (H) and cerebellum (CB). Protein quantitation is shown as the average (pg/mL) +/- SEM for (A) GFAP and (B) UCH-L1. (N = 5).

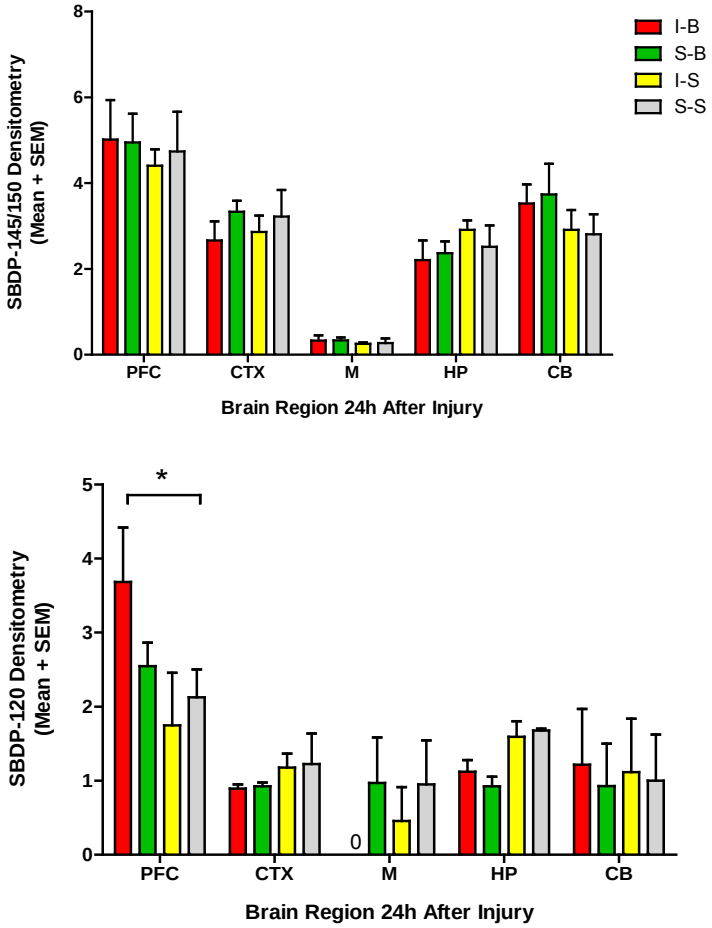


Figure 2. Semi-quantitative Western blotting of αII-Spectrin break-down products (SBDPs) as indicators of neuronal injury after fear conditioning and mTBI from BOP. Tissue samples are from rats in Task 3b and were sacrificed ~24 h following the last BOP exposure. Proteins were individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (PFC), cerebral cortex (CTX), midbrain (M), hippocampus (H) and cerebellum (CB). Protein band densitometry quantitation is shown as the average +/- SEM for (A) SBDP-145/150 and (B) SBDP-120. An asterisk indicates protein measurements that are significant between individual groups by 1-tailed t-Test ($p \leq 0.05$, N = 5).

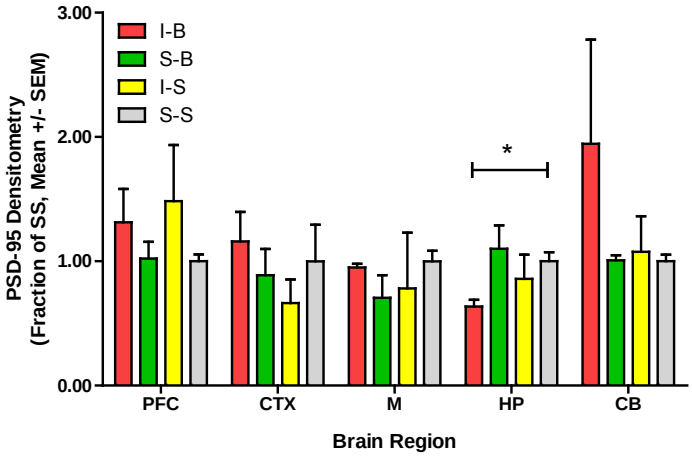


Figure 3. Semi-quantitative Western blotting of PSD-95, a major dendritic spine protein, as an indicator of neuronal dendritic spine injury after fear conditioning and mTBI from BOP. Tissue samples are from rats in Task 3b and were sacrificed ~24 h following the last BOP exposure. Proteins were individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (PFC), cerebral cortex (CTX), midbrain (M), hippocampus (H) and cerebellum (CB). The relative fold change of protein band densitometry quantitation is shown as the average +/- SEM. An asterisk indicates protein measurements that are significant between individual groups by 1-tailed t-Test ($p \leq 0.05$, $N = 5$).

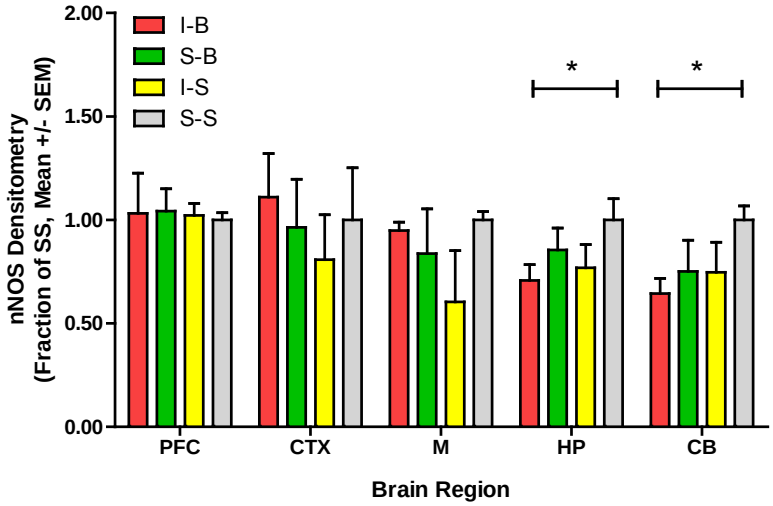


Figure 4. Semi-quantitative Western blotting of neuronal nitric oxide synthase (nNOS), a PSD-95 binding partner, as an indicator of neuronal injury after fear conditioning and

mTBI from BOP. Tissue samples are from rats in Task 3b and were sacrificed ~24 h following the last BOP exposure. Proteins were individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (PFC), cerebral cortex (CTX), midbrain (M), hippocampus (H) and cerebellum (CB). The relative fold change of protein band densitometry quantitation is shown as the average +/- SEM. An asterisk indicates protein measurements that are significant between individual groups by 1-tailed t-Test ($p \leq 0.05$, N = 5).

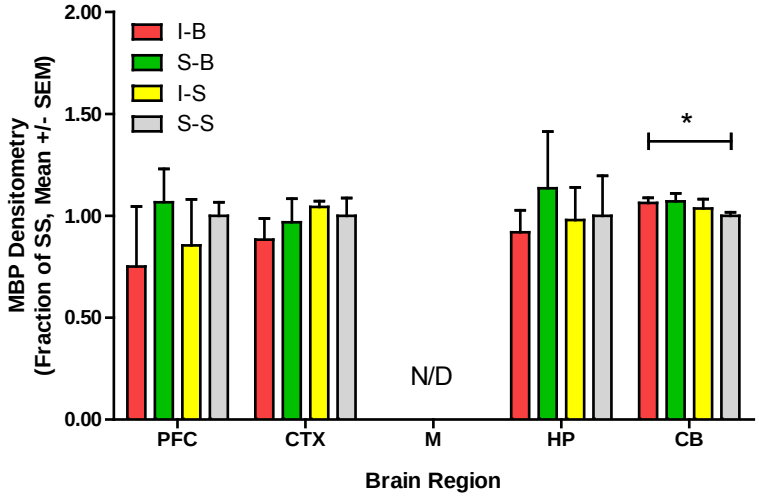


Figure 5. Semi-quantitative Western blotting of myelin basic proteins (MBP) as an indicator of white matter injury after fear conditioning and mTBI from BOP. Tissue samples are from rats in Task 3b and were sacrificed ~24 h following the last BOP exposure. Proteins were individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (PFC), cerebral cortex (CTX), midbrain (M), hippocampus (H) and cerebellum (CB). Analysis of midbrain is pending (N/D = no data). The relative fold change of protein band densitometry quantitation is shown as the average +/- SEM. An asterisk indicates protein measurements that are significant between individual groups by 1-tailed t-Test ($p \leq 0.05$, N = 3).

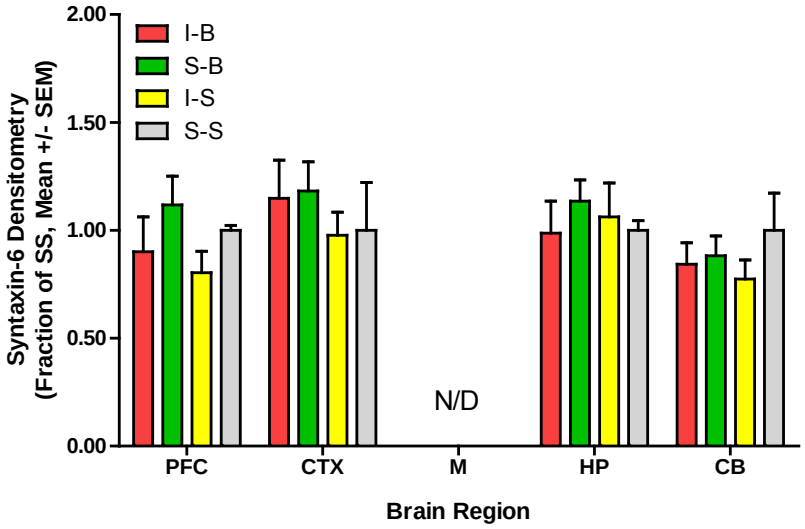
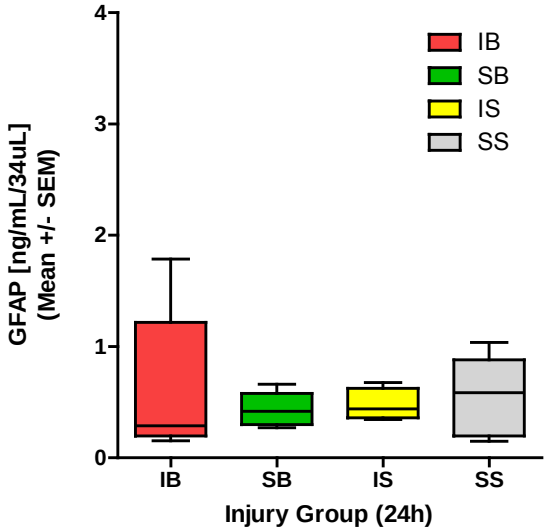


Figure 6. Semi-quantitative Western blotting of syntaxin-6 as an indicator of injury after fear conditioning and mTBI from BOP. Tissue samples are from rats in Task 3b and were sacrificed ~24 h following the last BOP exposure. Proteins were individually measured in clarified tissue lysates from specific brain regions: prefrontal cortex (PFC), cerebral cortex (CTX), hippocampus (H) and cerebellum (CB). Analysis of midbrain is pending (N/D = no data). The relative fold change of protein band densitometry quantitation is shown as the average +/- SEM. An asterisk indicates protein measurements that are significant between individual groups by 1-tailed t-Test ($p \leq 0.05$, N = 3).



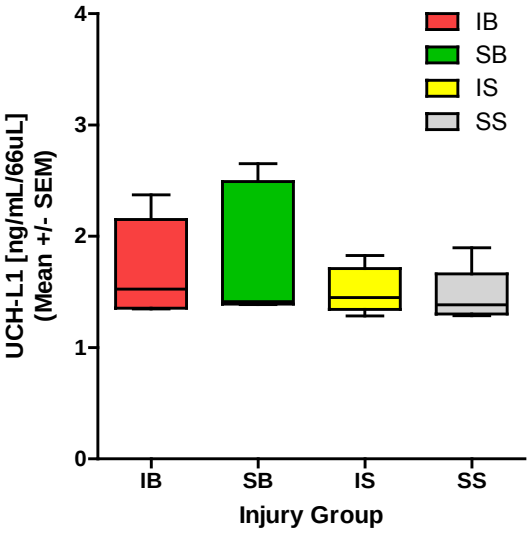
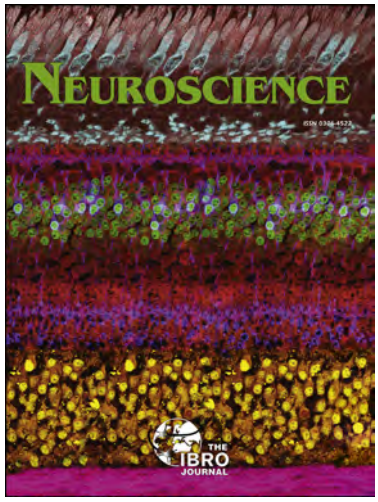


Figure 7. Quantitative ELISA of traumatic brain injury biomarkers after fear conditioning and mTBI from BOP. Serum samples are from rats in Task 3b and were sacrificed ~24 h following the last BOP exposure. Protein quantitation is shown as the average (ng/mL) +/- SEM for (A) GFAP and (B) UCH-L1 (N=5).



Effects of Mild TBI from Repeated Blast Overpressure on the Expression and Extinction of Conditioned Fear in Rats

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Effects of Mild TBI from Repeated Blast Overpressure on the Expression and Extinction of Conditioned Fear in Rats.[1]

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[1]Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting true views of the Department of the Army, Department of the Navy or the Department of Defense. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 and 2011 editions.

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ABSTRACT

Mild traumatic brain injury (mTBI) and post-traumatic stress disorder (PTSD) are pressing medical issues for the Warfighter. Symptoms of mTBI can overlap with those of PTSD, suggesting the possibility of a causal or mediating role of mTBI in PTSD. To address whether mTBI can exacerbate the neurobiological processes associated with traumatic stress, we evaluated the impact of mTBI from a blast overpressure (BOP) on the expression of a conditioned fear. In the rat, conditioned fear models are used to evaluate the emotional conditioning processes that are known to become dysfunctional in PTSD. Rats were first trained on a variable interval (VI), food maintained, operant conditioning task that established a general measure of performance. Inescapable electric shock (IES) was paired with an audio-visual conditioned stimulus (CS) and followed one day later by three daily exposures to BOP (75 kPa). Subsequently, the CS alone was presented once every seven days for two months, beginning four days following the last BOP. The CS was presented during the VI sessions allowing a concurrent measure of performance. Treatment groups (n=10, each group) received IES+BOP, IES+sham-BOP, sham-IES+BOP or sham-IES+sham-BOP. As expected, pairing the CS with IES produced a robust conditioned fear that was quantified by a suppression of responding on the VI. BOP significantly decreased the expression of the conditioned fear. No systematic short- or long-term performance deficits were observed on the VI from BOP. These results show that mTBI from BOP can affect the expression of a conditioned fear and suggests that BOP caused a decrease in inhibitory behavioral control. Continued presentation of the CS produced progressively less response suppression in both fear conditioned treatments, consistent with extinction of the conditioned fear. Taken together, these results show that mTBI from BOP can affect the

expression of a conditioned fear but not necessarily in a manner that increases the conditioned fear or extends the extinction process.

Keywords: mTBI, blast overpressure, conditioned fear, PTSD, stress processes, operant conditioning.

Abbreviations.

BOP, blast over pressure.

CS, conditioned stimulus.

CR, conditioned response.

IES, inescapable electric shock.

kPa, kilopascal.

mTBI, mild traumatic brain injury.

PTSD, post-traumatic stress disorder.

VI, variable interval schedule of reinforcement.

1.0 INTRODUCTION

Post-traumatic stress disorder (PTSD) and mild traumatic brain injury (mTBI) are significant health concerns for the Warfighter. A substantial percentage of individuals exposed to mTBI experience persistent symptoms (i.e., post-concussive syndrome) and both TBI and mTBI have been associated with psychiatric disorders including major depressive disorder (Vanderploeg, et al., 2007; Bombadier, et al., 2010). It has also been observed that mTBI is associated with the subsequent occurrence of PTSD (e.g., Hoge, et al., 2008). The occurrence of mTBI on the battlefield, however, is also typically associated with psychological trauma which further complicates the delineation. Nevertheless, the association and overlap in symptoms between mTBI and PTSD has raised the possibility that mTBI could mediate or, in some manner, predispose an individual to PTSD. While the neurobiology of such a relationship has not been demonstrated, some mechanisms have been proposed (Simmons and Matthews, 2012).

There are, however, only a few preclinical laboratory studies investigating the relationship between mTBI and animal models of PTSD. For example, Reger, et al. (2012) evaluated conditioned fear in rats several days after a fluid percussion injury. Using several conditioned freezing procedures, they reported an increased conditioned fear response (i.e., an increased freezing time) due to injury. Elder, et al. (2012) found behavioral changes in rats six weeks after mTBI from blast overpressure (BOP) which they interpret as an increase in "PTSD-like traits". The behavioral changes included decreased exploratory activity on an elevated plus maze, decreased open-field center activity following exposure to a predator cue, enhanced acoustic startle response and an enhanced conditioned fear. mTBI from a weight drop procedure has also been reported to decrease open-arm time in a plus maze without decreasing total distance (six days post injury) and to enhance a conditioned fear (eight days post injury), while

not affecting its extinction (Meyer, et al., 2012). Thus, evidence exists that mTBI can alter the conditioned fear process.

We further evaluated the relationship between mTBI and conditioned fear in rats. We were particularly interested in determining whether mTBI could alter the process of extinction to a conditioned fear. An integral feature of many of the emotional conditioning processes involved in PTSD is that the conditioned stimuli are resistant to extinction. That is, stimuli that are associated with traumatic events continue to elicit intense emotional responses despite their repeated presentations in the absence of traumatic events (American Psychiatric Association, 2000). Thus, in addition to evaluating whether mTBI would alter the magnitude of a conditioned fear, we were also interested in evaluating whether mTBI would alter the extinction function within the conditioned fear paradigm.

To evaluate a conditioned fear, we used the method of Estes and Skinner (1941). In our implementation, previously neutral audio-visual stimuli are paired with aversive inescapable electric shock (IES) and, subsequently, elicit a conditioned emotional response which is generally described as fear. After conditioning, the audio-visual stimulus is embedded in an operant task and the resulting response suppression reflects the strength of the conditioned fear. Thus, the procedure allows for the concurrent evaluation of the conditioned fear and the general performance on the operant task. We evaluated extinction by repeated presentation of the audio-visual stimulus without IES over weekly test sessions for two months. It is notable that the conditioned suppression model is in contrast to conditioned freezing methods. While freezing would necessarily constitute response suppression, studies have shown that the suppression model involves additional conditioned fear processes (Amorapanth et al., 1999; Lee, et al., 2005; McDannald, 2010; Pickens, et al., 2010; McDannald and Galarce, 2011).

Clinically, the defining characteristics for the classification of mTBI are almost entirely based on signs or symptoms and include a relatively broad range of severity (Defense and Veterans Brain Injury Center, 2006). The translation to an infrahuman equivalent of mTBI is, therefore, notably challenging and is further complicated by the number and diversity of laboratory models in use (see review by DeWitt, et al., 2013). In the present study, we used controlled exposure to blast overpressure to produce mTBI. This model is reasonably well characterized (e.g., Long, et al., 2009; 2010) and is particularly relevant as it closely represents a portion of the process resulting in a high prevalence of mTBI on the battlefield as documented from recent conflicts (Okie, 2005; Warden, 2006). In this regard, it is notable that BOP alone does not model the impact injuries that can accompany explosive blasts on the battlefield.

We chose a BOP of 74.5 kPa (10.9 psi) with the intention of producing an insult which could be considered to be in the low end of the mTBI range. The chosen pressure is less than those associated with gross pathology and specifically, neuronal pathology (e.g., Long et al., 2009; Readnower, et al., 2010; Kamnaksh, et al., 2011), but has been shown to produce behavioral effects such as anterograde amnesia (Ahlers, et al., 2012). Additionally, we used three BOP exposures (1/day). This regimen has been used previously and was not found to produce neuronal pathology, but was found to produce behavioral effects including increased startle response and decreased maze movement (Elder, et al., 2012). Finally, to exclude the traumatic stress that would be expected to accompany the BOP exposure, rats were anesthetized before both the BOP and sham exposures.

2.0 EXPERIMENTAL PROCEDURES

2.1 Animals.

This study was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition. All procedures were reviewed and approved by the Institutes' Animal Care and Use Committees, and performed in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

Adult male Sprague-Dawley rats (Charles River, Wilmington, MA) were used. Rats were individually housed in a temperature-controlled environment under a 12L:12D cycle (lights on at 06:00 h) and water was always available in the home cages. Body weights were maintained at approximately 325 g by food administered during experimental sessions and supplemental feedings (PMI Nutrition International, St. Louis, MO) occurring several hours after experimental sessions. All rats were weighed daily, Monday-Friday.

2.2 Variable interval schedule of reinforcement.

All rats were trained on a variable-interval 32 second schedule of reinforcement (VI). Sessions were conducted in twelve standard rodent operant conditioning chambers (model ENV-008 or equivalent, Med Associates, St. Albans, VT) housed in ventilated, light- and sound-attenuating cubicles. Each chamber contained two response levers and a food trough attached to a food dispenser capable of delivering 45 mg food pellets (F0021, Bio-Serv, Frenchtown, NJ). Each chamber also contained a house light mounted on the front wall near the ceiling, a stimulus light mounted above each of the response levers and a Sonalert® tone generator (~2.8 kHz, model ENV-223 AM or equivalent, Med Associates, St. Albans, VT). Experimental events were controlled and monitored by a microcomputer, using Med-PC® control software (Med Associates, St. Albans, VT).

Although two levers were present in each chamber, only one lever produced food reinforcement. In this regard, an equal number of boxes were designated with the active lever on the left and on the right. Rats were initially trained to lever-press for food pellets under a continuous schedule of reinforcement where one lever press on the active lever always produced one food pellet. When lever pressing was maintained by food presentation, the contingencies were changed to the VI. The VI specifies that a single lever-press, following an average interval of 32 sec, produces food reinforcement (i.e., a single food pellet). Interval values for the schedule were chosen pseudo-randomly, without replacement, from a set of values that followed a normal distribution (range=0.8-127.9 sec). Normal VI sessions were conducted without illuminating the house light or the stimulus lights above both levers. All sessions were 30 min in duration.

When responding under the VI was stable (as judged by inspection of the daily response rates), rats were assigned to a treatment group. In all cases, at least 60 training sessions were conducted before assignment. Assignment was balanced with respect to rate of responding with the objective that each treatment group would have similar average rates of responding.

2.3 Fear conditioning.

Conditioning took place in a stainless steel chamber measuring 109 cm x 66 cm x 97 cm placed inside a ventilated, sound- and light-attenuating cabinet. The chamber contained a grid floor consisting of stainless steel rods running along the width and electric shock stimuli were presented through these rods. Inescapable electric shock (IES) stimuli were generated by a Programmable Shocker (Lafayette Instrument Company, IN, model HSMSCK). The device was used to output an isolated, scrambled (4-pole), constant current, electrical stimulus calibrated to 1.0 mA. The onset and duration of the shock stimulus was controlled by a laptop computer and

associated interface equipment using custom software. The chamber also contained two house lights, four stimulus lights and a Sonalert ® tone generator, identical to those in the chambers used for the VI32 sessions.

Rats were placed in the chamber for a 35 min session in which 20 electric shock stimuli were presented at random times during the session with the exclusion of the first and last 2.5 min of the session and with at least 30 sec between presentations. Each shock stimulus was 1.0 mA in intensity and 2.0 sec in duration. A conditioned stimulus (CS) was presented 0.5 sec prior to the onset of the shock stimulus and continued for the duration of the shock stimulus (i.e., 2.5 sec total). The CS consisted of intermittently operating the Sonalert (0.35 sec on, 0.15 sec off), house light (0.25 sec on, 0.25 sec off) and stimulus lights (0.15 sec on, 0.35 sec off). For sham control conditions, sessions were conducted with all of the same stimuli and parameters except the shock stimuli were not presented.

2.4 Conditioned response (CR) testing.

Sessions for the CR testing procedure were conducted in an identical fashion as the VI sessions described in section 2.2 except that a CS (flashing lights and a pulsing tone) lasting 2.5 sec was presented once during the session. The CS presented was essentially identical to that used during the fear conditioning procedure described in section 2.3. That is, the CS consisted of intermittently operating the Sonalert (0.35 sec on, 0.15 sec off), house light (0.25 sec on, 0.25 sec off) and stimulus lights (0.15 sec on, 0.35 sec off) during the VI session. The CS was presented once during a session at a time randomly chosen from an array of times following a normal distribution but excluding the first 6 minutes and the last 8 min of the 30 min session.

2.5 Blast overpressure exposure**.**

Rats were exposed to overpressure using a shock tube and air blast exposure under controlled conditions. The shock tube has a 12-inch circular diameter and is a 17.5 ft long steel tube divided into a 2.5 ft compression chamber that is separated from a 15 ft expansion chamber. The compression and expansion chambers are separated by polyethylene Mylar ^{TM} sheets that control the peak pressure generated. The peak pressure at the end of the expansion chamber was determined by piezoresistive gauges specifically designed for pressure-time (impulse) measurements (Model 102M152, PCB, Piezotronics, Inc., Depew, NY, USA).

Rats were first anesthetized using an isoflurane gas anesthesia system consisting of a vaporizer, gas lines and valves, and an activated charcoal scavenging system. Rats were placed into a polycarbonate induction chamber, which was closed and immediately flushed with a 5% isoflurane mixture in air for two minutes. Rats were then placed into a cone-shaped plastic restraint device and then placed into the shock tube. Movement was further restricted during the blast exposure using restraint straps made from 1.5 cm diameter flattened rubber tourniquet tubing. Three such straps were spaced evenly to secure the head region, the upper torso and lower torso while the animal was in the plastic restraint cone. The end of each strap was threaded through a toggle and run outside of the exposure cage where it was tied to prevent movement during the blast overpressure exposure without restricting breathing. Rats were positioned with the head facing the blast exposure without body shielding to produce a full body exposure to the blast wave. Blast exposed animals received 74.5 kilopascal (kPa) exposures equivalent to 10.9 pounds per square inch (psi). Using this system, the duration of the overpressure has been determined to be ~4.8 msec (Ahlers, et al., 2012). Additionally, tests were performed to estimate variability in the maximum overpressure which was found to be 74.5 (±4.5) kPa, mean and SEM, respectively (Ahlers, et al., 2012). One exposure per day was administered for three consecutive

days. For sham control conditions, rats were treated identically, including anesthesia and restraint, but did not receive a blast exposure.

2.6 Experimental series, groups and treatments.

Extinction to a conditioned fear, with or without repeated BOP, was evaluated over the course of eight weeks (see Fig. 1). Additionally, performance on the VI was evaluated during daily (Mon-Fri) sessions over the same time period. Four treatment groups were used (n=10, each group): IES+Sham, IES+BOP, Sham+BOP, and Sham+Sham. All rats were first trained on the VI as described in section 2.2. Following training, rats were fear conditioned as described in section 2.3 by pairing IES with the CS (auditory and visual stimuli) for groups IES+Sham and IES+BOP. Sham control treatments for fear conditioning included the CS but without the IES (groups Sham+BOP and Sham+Sham). Fear conditioning took place during a single session following the VI session. On the three days following fear conditioning, rats in the treatment groups IES+BOP and Sham+BOP received exposure to BOP as described section 2.5. The exposures took place ~2 hours before the VI session. For treatment groups IES+Sham and Sham+Sham, sham-BOP exposures were delivered. Four days after the last BOP or sham-BOP exposure (seven days after the CS+IES or CS+sham-IES) a single CS was presented during the VI session (i.e., CR testing) for all treatment groups. VI sessions were continued Mon-Fri, with a single CS presented during the VI session conducted on Mondays, for a total of 8 CR tests. All rats were euthanized at the end of testing and tissue samples were taken as part of a larger proteomic study, to be presented separately.

2.7 Statistical analyses.

When a response (i.e., lever press) occurred during the VI, the elapsed time within the session was recorded. From these data, the total number of responses and the rate of responding (responses per min) were calculated for each rat for the "active" lever (i.e., the lever producing food reward) and the inactive lever. Responding on the inactive lever was always very slow, typically accounting for less than one percent of the responses, and did not change systematically throughout the experiment. Therefore, these data were not analyzed further. Response rates on the active lever from the six sessions before CS+IES (or CS+sham-IES) were averaged and treated as a baseline control. Response rate data from subsequent sessions were converted to a percentage of the baseline values for each rat (i.e., percent of control).

Forty VI sessions were conducted following the CS+IES (or CS+sham-IES) presentation and response rate measures from these sessions were averaged into eight blocks of five consecutive sessions for analysis. Additionally, individual sessions were analyzed after the CS+IES (or CS+sham-IES) presentation and up to the first CR test session. This period of performance included VI sessions conducted after each of the BOP (or sham-BOP) exposures.

To evaluate the strength of the CR for fear conditioning, suppression indices were calculated according to the formula: (response rate before the CS - response rate after the CS) / (response rate before the CS + response rate after the CS). This measure yields a value of 0 when there is no response suppression due to CS presentation and a value of 1 when responding is completely suppressed by CS presentation. We calculated suppression indices for both +/- 1 min and +/- 3 min around the CS. While we expected these measures to be correlated, their use maximized the quantification of the strength of the conditioned fear and also reduced the possibility of a restriction due to a ceiling effect that might occur if responding was completely

suppressed during a short interval following the CS. Suppression indices were calculated for each rat for each CR test session.

Inferential statistics were calculated using the SAS (Cary, NC) statistical software package. A two factor (treatment by time) mixed model ANOVA (with a Satterthwaite approximation for the denominator degrees of freedom) was performed for the VI response rate and suppression index measures. The procedure allows for the specification of the covariance structure. Based upon measures of fit (e.g., AIC, AICC and BIC), a compound symmetry model was used for the VI response rate data and an autoregressive model (AR1) was used for the suppression index data. Following ANOVA, selected contrasts were performed. In all cases, the criterion for statistical significance was set at $p<.05$.

3.0 RESULTS

Responding under the VI schedule was acquired by all rats. Baseline measures of responding on the active lever (i.e., the lever producing food reinforcement), defined as the average of the last 6 sessions conducted before exposure, for the treatment groups (n=10 each group) were as follows (mean ± SEM responses per min): IES+Sham=51.4 ± 6.9, IES+BOP=56.5 ± 10.3, Sham+BOP=50.1 ± 3.7, Sham+Sham=50.7 ± 5.1. Fig. 2 shows performance on the VI from the last baseline session through the first CR test. ANOVA evaluating VI performance during the five sessions after fear conditioning and including the session with the first CR test revealed no significant effects for group ($F[3,36]=0.96$, $p>.05$) or the group by session interaction ($F[12,144]=0.75$, $p>.05$) but did reveal a significant main effect for session ($F[4,144]=8.71$, $p<.001$). Tests of effect slices for the session factor showed significant effects for the IES+BOP ($F[4,144]=3.32$, $p<.01$) and Sham+Sham ($F[4,144]=3.96$,

p<.01) treatment groups but not for the IES+Sham (F[4,144]=1.38, p>.05) or Sham+BOP (F[4,144]=2.31, p>.05) groups. Although there was not a significant main effect for groups, we were particularly interested in whether any changes could be attributed to a common treatment of IES or BOP presentation. Thus, we evaluated, but found no significant effects for, contrasts comparing groups receiving BOP (IES+BOP and Sham+BOP) vs. no BOP (IES+Sham and Sham+Sham) (F[1,36]=1.83, p>.05), and IES (IES+BOP and IES+Sham) vs. no IES (Sham+BOP and Sham+Sham) (F[1,36]=1.06, p>.05).

Fig. 3 shows VI performance over eight weeks beginning with the session following CS+IES (or CS+sham-IES). In general, performance on the VI was maintained near baseline levels in all groups, although some deviations from baseline were present. ANOVA showed no main effects for groups (F[3,36]=1.77, p>.05) and no groups by session interaction (F[21,252]=1.21, p>.05), but did show a significant main effect for sessions (F[7,252]=2.07, p<.05). Analysis of effect slices for sessions revealed a significant effect only for the IES+Sham group (F[7,252]=2.44, p<.05). No significant effect was found for contrasts that compared IES groups vs. no IES groups (IES+Sham and IES+BOP vs. Sham+BOP and Sham+Sham, F[1,18]=.03, p>.05) or BOP groups vs. no BOP groups (IES+BOP and Sham+BOP vs. IES+Sham and Sham+Sham, F[1,18]=5.03, p>.05).

No grossly observable effects from the IES or from BOP exposures were noted. All rats appeared normal shortly following anesthesia and BOP and shortly following IES. Furthermore, all rats appeared normal during weighing and handling throughout the experiment. Fig.4 presents the extinction functions for conditioned fear, as evidenced by the degree of response suppression (suppression index for +/- 1 min [top] and +/- 3 min [bottom]), for the four treatment groups during the eight consecutive weekly CR tests. As expected, presentation of the CS during

the VI session initially produced substantial response suppression in treatment groups where the CS had been previously paired with IES (i.e., IES+Sham and IES+BOP). Also as expected, the CS produced very little response suppression in treatment groups where the CS had not been paired with IES. In general, when present, the response suppression produced by the CS was greater for the +/- 1 min index as compared with the +/- 3 min index. For the +/- 1 min suppression index (Fig. 4, top panel), ANOVA showed a significant main effect for treatment group ($F[3,82.6]=22.62$, $p<.001$), CR session ($F[7,180]=10.22$, $p<.001$) and the treatment group by CR session interaction ($F[21,180]=2.87$, $p<.001$). Similarly, for the +/- 3 min suppression index (Fig. 4, bottom panel), ANOVA showed a significant main effect for treatment group ($F[3,61.3]=9.95$, $p<.001$), CR session ($F[7,180]=6.82$, $p<.001$) and the treatment group by CR session interaction ($F[21,180]=3.78$, $p<.001$). For both the IES+Sham and IES+BOP treatment groups, the conditioned fear diminished with continued presentation of the CS as can be seen by a reduction in both suppression indices during the later CR test sessions. Analyses of the effect slices for CR sessions for the +/- 1 min suppression index showed significant effects for both the IES+Sham ($F[7,180]=11.39$, $p<.001$) and IES+BOP ($F[7,180]=6.80$, $p<.001$), but not for the Sham+BOP ($F[7,180]=.31$, $p>.05$) and Sham+Sham ($F[7,180]=.034$, $p>.05$). The same profile of significance was found for the +/- 3 min suppression index (IES+Sham, $F[7,180]=14.82$, $p<.001$; IES+BOP, $F[7,180]=2.66$, $p<.02$; Sham+BOP, $F[7,180]=.49$, $p>.05$; Sham+Sham, $F[7,180]=.17$, $p>.05$). As can be seen from Fig. 4, conditioned fear in the IES+Sham group was, on average, greater than that in the IES+BOP group. Contrasts between these two groups across CR sessions showed a significant difference for both the +/- 1 min index ($F[1,82.6]=9.24$, $p<.005$) and the +/- 3 min index ($F[1,61.3]=6.42$, $p<.02$). The difference between these two groups is also illustrated in Fig. 5 which shows the degree of suppression (suppression index for +/- 1 min [top] and +/- 3

min [bottom]) for the four treatment groups during the first CR test. Contrasts comparing the treatment groups at this time point show a significant difference in suppression between the IES+Sham-S and IES+BOP treatments for both the +/- 1 min (top panel, $F[1,268]=7.54$, $p<.01$) and the +/- 3 min (bottom panel, $F[1,223]=26.54$, $p<.001$) indices. Additionally, both the IES+Sham and IES+BOP groups were significantly different than both the Sham+BOP and Sham+Sham groups for the +/- 1 min ($Fs[1,223]\geq21.48$, $ps<.001$) and the +/- 3 min ($Fs\geq10.75$, $ps<.01$) indices. The Sham+BOP and Sham+Sham groups did not differ significantly for either index (+/- 1 min index, $F[1,268]=.22$, $p>.05$; +/- 3 min index, $F[1,223]=.30$, $p>.05$).

4.0 DISCUSSION

We trained rats on an operant schedule of food reinforcement and then trained a conditioned fear by pairing IES with an audio-visual stimulus. Subsequently, rats were exposed to repeated mTBI from BOP. Presentation of the CS after pairing with IES took place in a different context than the IES and produced a robust conditioned fear as quantified by both the +/- 1 min and +/- 3 min suppression indices. BOP reduced the degree of conditioned suppression. That is, as compared to sham-BOP controls, BOP decreased the expression of a conditioned fear that was trained prior to exposure. There are several possible interpretations of this result. First, it could be argued that the BOP produced sensory damage to the auditory and/or visual system such that the perception of the CS was altered in exposed rats. While BOP, using a similar procedure as in the present study, has been reported to produce visual system degeneration, it did so only at substantially higher pressures (104-173 kPa) and a pressure of 84 kPa, which is greater than that used in the present study, did not result in any visual system pathology (Petras, et al., 1997). Additionally, the exact regimen of BOP used in the present study was not found to

produce any changes in the prepulse inhibition of a startle response, suggesting that auditory perception was also not impaired (Elder, et al., 2012). Therefore, it is not likely that sensory damage due to BOP was responsible for the observed difference between the IES+Sham and IES+BOP treatment groups.

A second interpretation of this result is that the BOP produced a retrograde amnesia. In this regard, it is notable that the BOP exposures took place beginning at ~22 hours after the IES. It is likely that enough time had elapsed for memory consolidation of the event to have occurred (McGaugh, 2000). Thus, the amnesic effect would not have been through the disruption of memory consolidation processes such as when the insult takes place shortly after the conditioning event. Furthermore, a single BOP at the same and at a greater pressure than used in the present study did not produce an amnesic effect when exposure immediately followed a passive avoidance task (Ahlers, et al., 2012). Typically, more severe injuries are required to produce a retrograde amnesia for events already presumed to be consolidated into long term memory (e.g., Chen, et al., 2009). It is also notable that BOP exposed animals did show a conditioned fear, although to a lesser degree than the sham-BOP treatment group. Thus, the retrograde amnesia would have to be characterized as partial.

While a retrograde amnesia cannot be ruled out, we propose that the BOP exposure more likely decreased behavioral inhibition. That is, responding on the VI task is maintained by food reinforcement and the schedule of reinforcement exerts a degree of stimulus control (i.e., represents a motivated task). Following pairing with the IES, the CS elicits a conditioned response (i.e., conditioned fear) which is in conflict with responding on the VI task. In this sense, the CS serves as an inhibitory or "stop" signal. The BOP exposure appears to have decreased the inhibitory control exerted by the CS although responding on the VI was unaffected. While

further studies are needed to confirm this possibility, it is notable that failures of inhibitory control behaviors are integral features of many psychiatric disorders and the mechanism of an inhibitory control system in rats has been the subject of substantial study (see review by Eagle and Baunez, 2010). Furthermore, deficits in behavioral inhibition have been observed in patients following TBI (Dimoska-Di Marco, et al., 2011; Dockree, et al., 2006; O'Keeffe, et al., 2007).

The decreased expression of a conditioned fear produced by BOP in the present study represents a functional deficit. That is, the optimal conditioned fear response is best represented by the IES+sham treatment and a substantial deviation from that response can reasonably be interpreted as an adverse outcome. Our results, however, are in stark contrast to previous results showing that mTBI produced an exaggerated conditioned fear as compared to controls (Elder, et al., 2012; Meyer, et al., 2012; Reger, et al., 2012). A major difference between the former studies and the present study is that the conditioned fear in the present study was established (and presumably consolidated into long-term memory) before the mTBI exposures. In the former studies, conditioning took place days or weeks after the mTBI insults. A second difference is that we used a conditioned suppression procedure to evaluate conditioned fear whereas the former studies used a conditioned freezing procedure. Freezing, by definition, prevents lever pressing and previous studies have shown that conditioned freezing and conditioned suppression are correlated (e.g., Pickens, et al., 2010). Additional studies using lesioning have shown that the dependencies of these two behaviors on the basolateral and central nuclei of the amygdala, and the ventral periaqueductal grey differs (Amorapanth et al., 1999; Lee, et al., 2005; McDannald, 2010;; McDannald and Galarce, 2011). Therefore, the different results in the present study may reflect qualitatively different effects of mTBI on conditioned fear.

We were particularly interested in whether BOP would alter the course of extinction, independently of whether it affected the initial expression of conditioned fear. In this regard, both IES+Sham and IES+BOP rats showed orderly extinction functions (Fig. 4) that appeared to be essentially parallel. Repeated exposure to the CS produced progressively less suppression in both groups. There was a statistically significant difference in the extinction functions between the two groups, but that difference is consistent with the decreased initial conditioned fear response produced by BOP. Furthermore, both groups reached near zero values for the suppression indices that were equivalent to groups that had not received CS+IES pairing. We, therefore, conclude that BOP did not delay or facilitate extinction to a conditioned fear, although it did alter the magnitude of its expression. This result is consistent with a previous study (Meyer, et al., 2012) that showed that mTBI from weight drop did not alter extinction of a conditioned fear, while altering (in this case increasing) the initial expression of the conditioned fear. The finding is relevant to understanding the relationship between mTBI and PTSD since the persistence of an emotional response to previously neutral stimuli that have been associated with trauma is an integral feature of some PTSD symptoms (American Psychiatric Association, 2000). Additionally, individuals with PTSD have shown resistance to extinction in fear conditioning in the laboratory (e.g., Blechert, et al., 2007; Wessa and Flor, 2007). In this regard, our results do not support a predisposing or causative relationship between mTBI and PTSD.

Neither the BOP nor IES manipulations had a robust or systematic effect on responding on the VI. In general, responding was maintained during sessions occurring only a few hours after the BOP sessions. Previous studies have shown that operant behavior is a reasonably sensitive measure of performance and we have previously used such procedures to evaluate the effects of drugs, toxins and ischemic injury (Genovese, et al., 1988; Genovese, et al., 1992;

Genovese, et al., 1993; Genovese, et al., 2006). Moreover, TBI, from a fluid percussion injury, has been shown to decrease responding on a food-maintained operant task (Gorman, et al., 1993). The mTBI exposure in the present study was clearly insufficient to disrupt performance on the task taking place several hours later and clearly suggests that the intensity of the repeated BOP used was, in fact, a mild insult. We also did not observe the BOP to produce any long-term or delayed disruption on the VI task as we evaluated performance for approximately two months following the BOP. In this respect, we did not observe any delayed neurobehavioral effects known to occur in patients following TBI (e.g., Gualtieri and Cox, 1991), further suggesting that the BOP used in the present study is a mild insult.

As expected, rats that did not receive IES did not show any conditioned fear. The occurrence of the CS during the VI session, however, did produce a small degree of disruption in groups not receiving IES (Sham+BOP and Sham+Sham) as shown by suppression indices that were consistently above zero. CS presentation, however, constituted an abrupt and unpredictable disruption of the normally dark and quiet VI environment. In this regard, the response to the CS in rats not previously receiving CS+IES pairings can be considered a startle response. There was no difference in this startle response, however, between groups receiving BOP or sham-BOP. It is also notable that the anesthesia used in the present study did not appear to affect any of the performance measures. Isoflurane was used to eliminate any traumatic stress from the BOP and also to reduce any movement that might produce variability in the effects of the BOP, but the same anesthetic regimen was used in all treatment conditions.

The present study demonstrates that, in rats, a behavioral deficit in the expression of conditioned fear can be caused by mTBI from BOP. The BOP intensity used to produce mTBI was below that shown previously to produce gross pathology and specifically, neuronal

pathology and can be considered a mild insult. While the BOP produced a deficit in the expression of a conditioned fear, the deficit was observed as a reduction in the impact of the conditioned fear as compared to IES+sham controls. Taking into account the difference in initial impact, extinction to the conditioned fear appeared to occur normally in both IES+BOP and IES+Sham groups. It is notable that these results, demonstrating that the mTBI produced a reduction in the expression of a conditioned fear while not increasing resistance to extinction, are not in the direction of an effect that is analogous to PTSD. While affecting the conditioned fear, the BOP did not produce acute deficits on general performance as measured by the VI, even when evaluated hours after exposure. Furthermore, no delayed effects on the VI were caused by the mTBI as no systematic changes in long-term performance on the VI were observed over two months of post-mTBI evaluations. In this regard, the results suggest that the intensity of the mTBI was, indeed, mild and is likely at the lowest end of the mTBI continuum. Results from this study are, of course, limited to a single conditioned fear procedure and further research is required to extend the findings to additional behavioral process that are integral to animal models of PTSD. Taken together, however, these results augment previous studies evaluating the behavioral effects of mTBI and particularly the possible relationship between mTBI and PTSD.

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and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 and 2011 editions.

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Figure Legends

Figure 1. Study design. All rats were first trained on the variable interval schedule of reinforcement (VI) and VI sessions continued to be conducted daily (Mon-Fri) throughout the study. Fear conditioning was implemented by pairing an auditory and visual conditioned stimulus (CS) with inescapable electric shock (IES). Following conditioning, exposures to blast overpressure (BOP) were presented on three consecutive days. Tests to evaluate the conditioned response (CR) from fear conditioning were performed by presenting the CS once during the VI session and measuring the resulting degree of response suppression. CR test sessions were conducted every seven days after fear conditioning during the course of eight weeks.

Figure 2. Performance on the VI schedule of reinforcement during 6 consecutive test sessions. CS + IES (or sham) occurred following the test session on day 1. BOP (or sham) occurred ~2 h before the test sessions on days 2-4. The CS alone was presented during the session on day 8. Ordinate: Response rate as a percentage of control (determined as the average response rate from six baseline sessions). Abscissa: Consecutive days. Each point represents the mean (+/- SEM) from 10 rats. Dashed horizontal line indicates control rate of responding. Points to the left of the vertical dashed line represent the last baseline session.

Figure 3. Performance on the VI schedule during 8 consecutive weeks. Ordinate: Response rate as a percentage of control (determined as the average response rate from six consecutive baseline sessions). Abscissa: Consecutive blocks. Each point represents the mean (+/- SEM) from 10 rats

and each block contains the average response rate from five sessions. Dashed horizontal line indicates control rate of responding.

Figure 4. Extinction of conditioned suppression. Ordinates: Suppression indices (+/-1 min, top and +/-3 min, bottom) during eight weekly CR test sessions. Abscissas: Consecutive weeks. Each point represents the means (+/- SEM) from 10 rats. Dashed horizontal lines represent a suppression index value of 0 indicating the same rate of responding before the CS as after the CS (i.e., no response suppression).

Figure 5. Conditioned suppression during the first CR test administered seven days after CS + IES pairing and four days after the last BOP exposure. Ordinates: Suppression indices (+/-1 min, top and +/-3 min, bottom). Abscissas: Four treatment groups: IES + Sham , IES + BOP , Sham + BOP and Sham + Sham. Bars represents the mean (+SEM) from 10 rats. Asterisks indicate a statistically significant difference (contrasts following ANOVA, ps<.05) and "ns" indicates comparison not statistically significant.

Figure 1

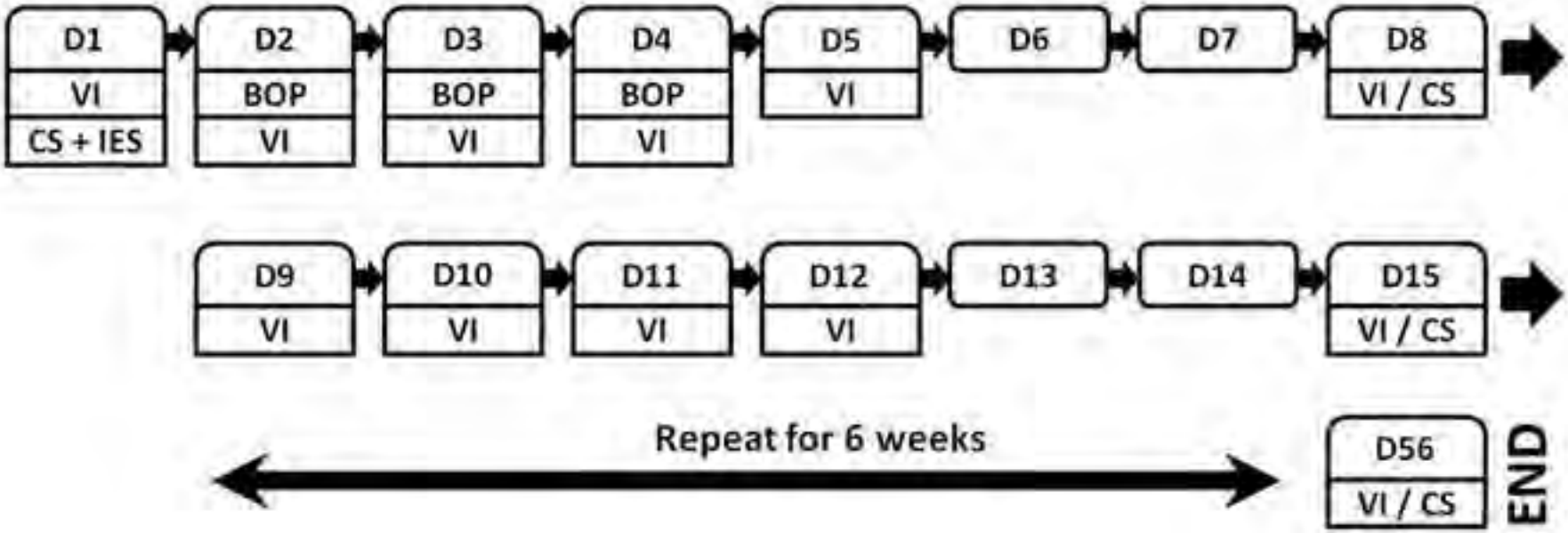


Figure 2

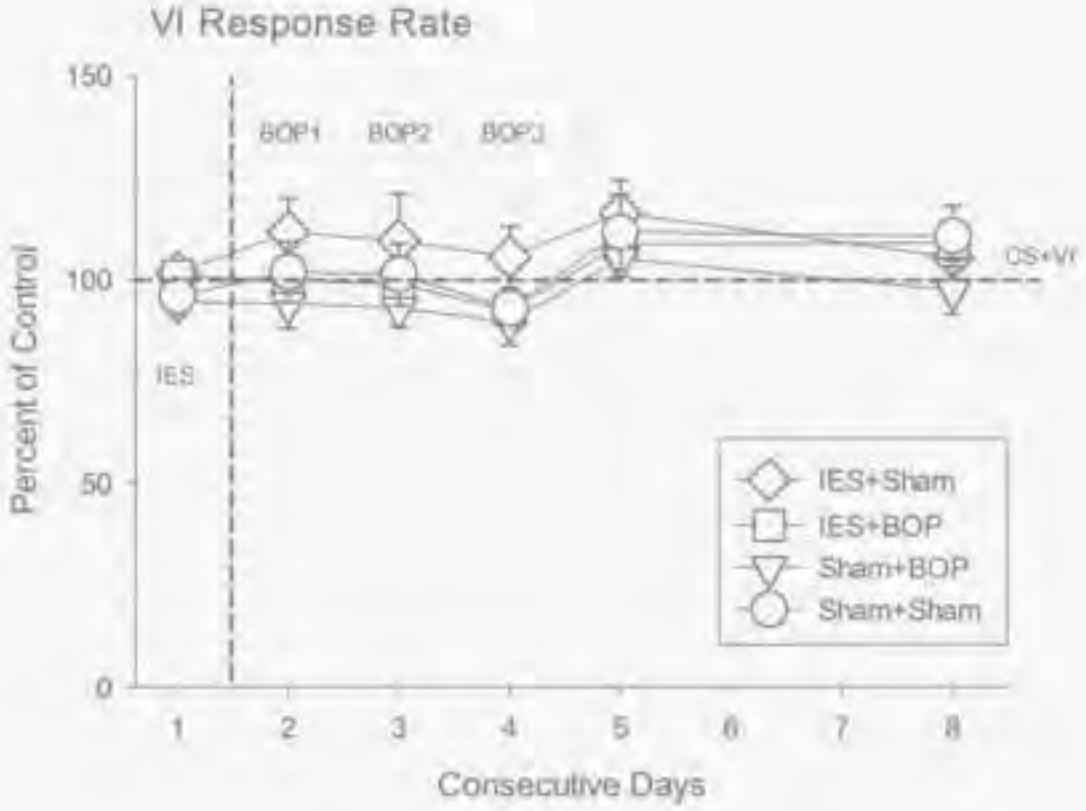


Figure 3

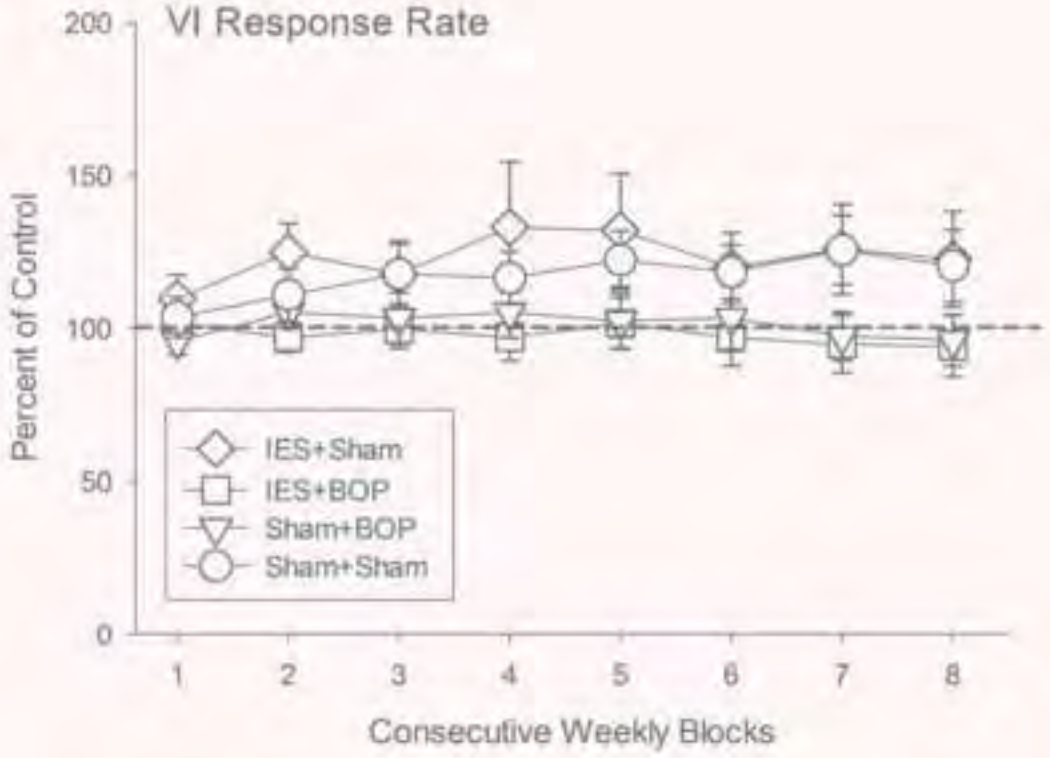


Figure 4

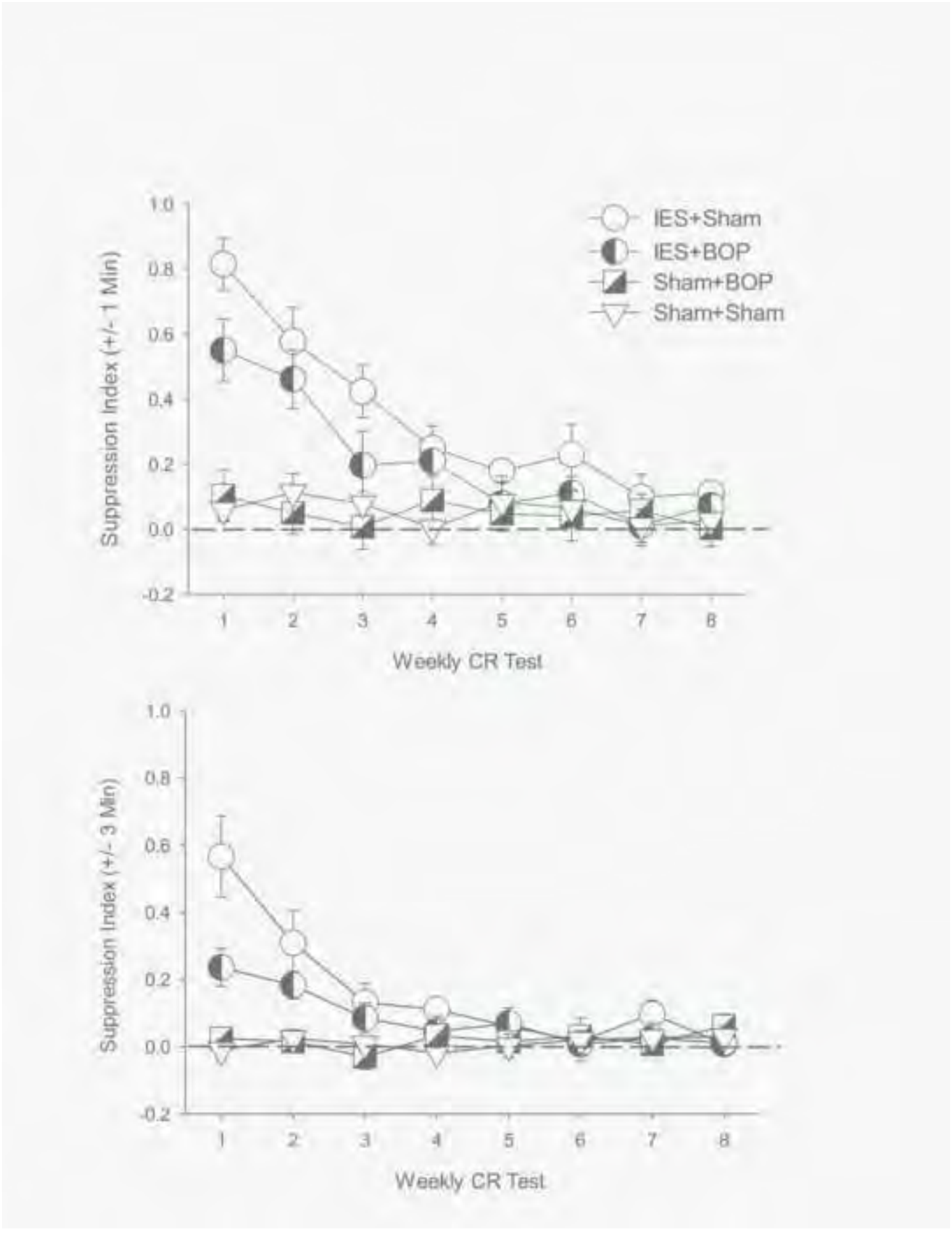
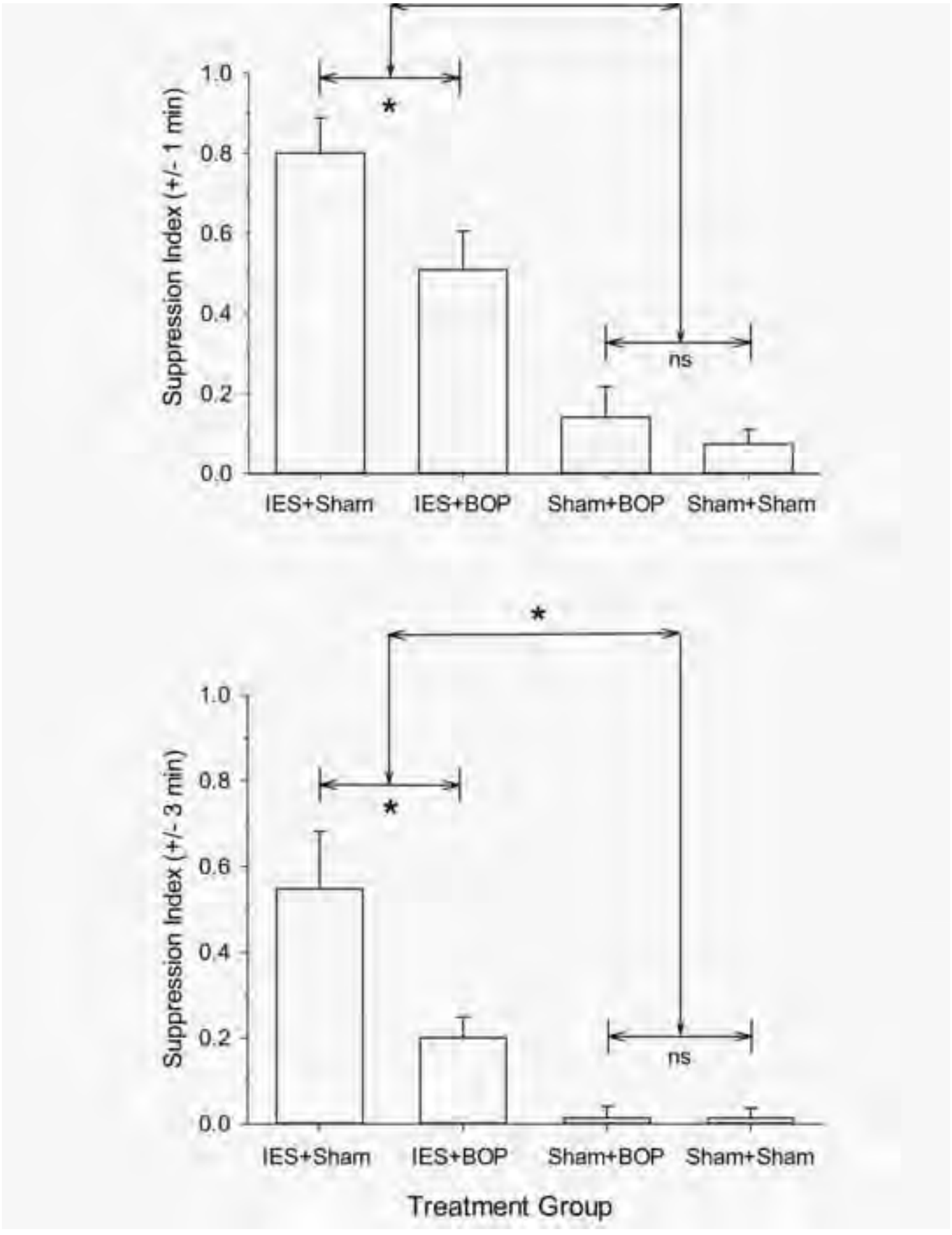


Figure 5



In rats, we evaluated the impact of mild TBI on conditioned fear. > Conditioned fear was implemented using a conditioned suppression procedure. > Mild TBI was produced through multiple blast overpressure exposures. > Mild TBI blunted the expression of a conditioned fear while not delaying extinction. > Results are relevant to a possible relationship between mTBI and PTSD.